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Cell Culture Studies with the IMC-Hz-1 Nonoccluded Virus

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16. ABSTRACT

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Cell Culture Studies with the IMC-Hz-1 Nonoccluded Virus[1]

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Studies were conducted on an adventitious agent (Hz-1V) isolated from the IMC-Hz-1 cell line. It appeared identical to the virus first obtained by Granados *et al.* (R. R. Granados, T. Nguyen, and B. Cato, 1978, *Intervirology*, **10**, 309–317) from a persistent infection of this cell line. Restriction endonuclease digestion of Hz-1V DNA indicated the agent was different from the S nuclear polyhedrosis virus (HzSNPV) of the host species, *Heliothis zea*, from which the IMC-Hz-1 cell line was derived by Hink and Ignoffo (W. F. Hink and C. M. Ignoffo, 1970, *Exp. Cell Res.* **60**, 307–309). Hz-1V caused extensive cytopathic effects (CPE) in cultures of TN-368 cells and was moderately infectious to several other insect cell lines. The lytic nature of the infection permitted development of a plaque assay employing TN-368 cells. Following acute infections of TN-368 cells, persistently infected cultures were readily established by the surviving cells. Attempts to induce persistent infections of clones 10 and 13 of TN-368 were, however, unsuccessful. Infectious Hz-1V was shed continuously into the medium throughout subculturings of persistently infected cells. The infectious titer dropped sharply during initial subculturings, but subsequently reached a basal level at about the 25th passage. These cultures were resistant to superinfection by homologous Hz-1V, but remained as susceptible as normal TN-368 cells to heterologous *Autographa californica M* NPV (Ac*M*NPV).

INTRODUCTION

A persistent infection of the IMC-Hz-1 cell line by a baculovirus-like agent was first reported by Granados *et al.* (1978). Cytopathic effects caused by the virus were observed by phase contrast and electron microscopy. Several cell lines, and especially TN-368, were found to be susceptible to this virus. Inoculation of larvae of Lepidopterous species by intrahemocoelic injections and feeding gave no biological or morphological indication of infection.

The presence of this agent in the IMC-Hz-1 cell line was first revealed by Granados at a symposium held on the use of viruses as biological pesticides (see Summers and Kawanishi, 1978, p. 177). Subsequent tests demonstrated that cultures of this cell line in our laboratory harbored the agent. Its presence in a cell line of *Heliothis zea*, the host species of a *S* nuclear polyhedrosis virus, which is the active ingredient of an EPA-registered "biorational pesticide" (Federal Register Notice, 1979, Vol. 44, No. 94), prompted the investigation of the relationship between these two viruses. Studies conducted in this context led to basic findings concerning the characteristics of Hz-1V and are the content of this paper.

MATERIALS AND METHODS

Cell lines. Studies were conducted with the *Trichoplusia ni* continuous cell line,

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TN-368, and two of its clonal sublines, clone 10 (Cl 10) and clone 13 (Cl 13) obtained by Volkman and Summers (1975). The persistently infected IMC-Hz-1 cell line was obtained from D. A. Goldstein, Scripps Clinic and Research Foundation. *Mamestra brassicae*, *Manduca sexta*, and *Spodoptera frugiperda* IPLB-21 cell lines were obtained from M. D. Summers, Texas A & M University. IPLB-21-1075 (sent to us as IPLB-1075 but later found to have an isozyme pattern of *S. frugiperda* cells), and BTI-EAA cell lines were obtained from R. R. Granados, Boyce Thompson Institute. Cell cultures were adapted to and maintained on TNM-FH medium of Hink (1970). Persistently infected TN-368 cultures were obtained as described below under Results.

Viruses. The virus, first designated IMC-Hz-1-NOV by Granados *et al.* (1978), is referred to here as Hz-1V for the sake of brevity. This agent was isolated by inoculating TN-368 cultures with 4-day growth medium from IMC-Hz-1 cell cultures as described by Granados *et al.* (1978). After 10 to 14 days, with subculturing as needed, the TN-368 cultures developed CPE. The harvested growth medium was clarified by low-speed centrifugation and Hz-1V infectivity titered by a plaque assay described below. Aliquots were frozen at −85° for future use.

The extracellular budded form of plaque-purified Ac*M*NPV E-2, a restriction endonuclease-characterized isolate of the *Autographa californica* nuclear polyhedrosis virus reported on by Smith and Summers (1979), was used in tests of superinfectivity of persistently infected cultures.

Plaque assay. The plaque assay utilizing TN-368 monolayers was a modification of Brown and Faulkner's technique (1978). A 1.5% overlay was prepared by autoclaving 3% Seaplaque agarose in distilled water, cooling to 35°, and adding an equal volume of 2× TNM-FH medium with 20% fetal bovine serum. Dishes (35 mm) were scored for plaques after 4 days at 28° with high humidity and a 5% CO_2 atmosphere. Both Hz-1V and Ac*M*NPV E-2 produce fairly large plaques, so removal of the overlay or observation with a microscope was not necessary. Counts were done in a darkened room with the plate illuminated with a narrow intense beam of light from below. These were the conditions of the standard assay utilized for all infectivity titrations reported in this study except where specifically noted.

When Cl 13 cells were used in the assay, the attachment time was extended to 24 hr because they adhered poorly to culture dishes. These dishes were neither rocked nor tilted during the 1-hr viral adsorption period, and agarose was overlaid in two stages to minimize cell detachment. The distribution of plaques did not indicate any abnormality due to these modifications.

The quantitative nature of the dose response of the plaque assay was tested in the following way. Supernatants from TN-368 cultures infected with different Hz-1V isolates were clarified as described previously. Each was diluted with medium to the point where discrete plaques could be reliably counted on the plates. These suspensions were considered the stock suspensions. The stock suspensions were further diluted in increments to a maximum of 1:10. Eight replicates were prepared for each dilution. Culture dishes were inoculated as before and incubated. Plaques were counted for each plate and the mean number of plaques for each dilution and confidence limits were calculated. This was done for each of the stock suspensions. Uninoculated control dishes were run for each assay.

Virus growth curve. TN-368 cells in logarithmic growth were seeded into eighteen 25-cm^2 culture flasks at a density of 1×10^6 cells and permitted to attach at 28° for 4 hr. Culture medium was removed from the flasks and half of the cultures were inoculated with 1-ml aliquots of Hz-1V at a multiplicity of infection (m.o.i.) of 5. The remaining flasks were mock infected with 1 ml of sterile culture medium. After incubation intervals of 1, 6, 12, 18, 24, 36, 48, 72, and 96 hr media from one treated culture and one control were clarified and stored as before. The cells were immediately checked for their viability using trypan blue exclusion as the crite-

rion. The number of plaque-forming units per milliliter (PFU/ml) was determined for each sample.

Susceptibility of cell lines. The susceptibility of different cell lines to Hz-1V was tested. Log phase cultures of TN-368, Cl10, Cl13, IPLB-21, IPLB-21(1075), BTI-EAA, *M. brassicae*, and *M. sexta* cells were inoculated at an approximate m.o.i. of 0.1. These cultures and controls were checked subsequently for CPE and the infectious titers of the media were determined after 2 and, in some cases, 5 days incubation at 28°.

Comparison of the efficiencies of TN-368 and Cl13 as indicator cells for the plaque assay. The relative plaque-forming efficiencies of monolayers of these two cell types were determined with the objective of enhancing the sensitivity of the assay for Hz-1V. Replicates (12–24 per experiment) of nearly confluent monolayers of each cell type were inoculated concurrently with common virus dilutions. Different Hz-1V isolates were utilized in the three experiments to offset possible virus effects. The plaque assay procedures with TN-368 and Cl13 were described earlier.

Induction and maintenance of persistently infected cell cultures. Persistently infected cultures of TN-368 were established and maintained as described in the appropriate sections under Results.

Resistance of persistently infected cultures to homologous superinfection. Uninfected TN-368 cells and a persistently infected line established from it (HA-38) were tested for their susceptibility to Hz-1V and Ac*M*NPV-E2. Groups consisting of nine flasks each were seeded with 1×10^6 TN-368 or HA-38 cells from log phase cultures. Three groups were prepared of TN-368 and three of HA-38 cells. One group (nine flasks) of each type of cell was inoculated (m.o.i. = 1) with Hz-1V, a second group with Ac*M*NPV-E2 and the third mock infected with an equivalent volume of media as controls. They were held at 28° and subsequently, a flask was removed from each of the six groups after 1, 6, 12, 18, 24, 36, 48, 72, and 96 hr. incubation. The culture medium from each flask was centrifuged to remove cells and large debris before storage at −85°. These samples were later titered for infectivity as before.

Electron microscopy. Both TN-368 cells infected with Hz-1V and cells from persistently infected cultures were prepared for examination as follows. Low-speed pellets of saline-washed cells were dispersed in the fixative of Karnovsky (1965) and held at room temperature for 0.5 hr. Fixation was continued with fresh fixative for an additional 4 hr at room temperature. Cells were rinsed in 0.1 *M* sodium cacodylate buffer, pH 7.4, and cell clumps were then congealed in thin layers of warm agar and minced. Postfixation was in 0.05 *M* cacodylate-buffered 1% osmium for 1 hr. Cells were then washed with distilled water, *en bloc* stained with aqueous 1% uranyl acetate, dehydrated in ethanol and propylene oxide, embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate, and examined with a Philips EM 300 at 60 kV.

DNA purification and digestion with restriction endonuclease XhoI. The *Xho*I restriction patterns are presented for comparison with Hz-1V isolates obtained in other laboratories and to differentiate it from Hz*S*NPV. DNA from virions pelleted from cell-free Hz-1V-infected TN-368 culture media and alkali-solubilized Hz*S*NPV polyhedra solutions were obtained as follows. Pellets were resuspended (1 mg/ml) in 1× SSC, made 1% with respect to Sarkosyl and digested with protease K (0.5 mg/ml, 4 hr, 37°). The DNAs were isolated by two cycles of CsCl gradient (mean density of 1.7 g/ml) centrifugation (35,000 rpm, Beckman T65, 72 hr, 20°) followed by fractionation. The DNA fractions were collected, dialyzed overnight in 0.1× SSC at 4°, and stored (−20°) before use.

Ten units of *Xho*I (Bethesda Research Laboratories) and 0.1 μg of Hz-1V or Hz*S*NPV DNA were incubated together at 37° for 6 to 8 hr. The pH 7.4 digestion buffer contained 10 m*M* Tris, 10 m*M* $MgCl_2$, 6 m*M* β-mercaptoethanol, and 60 m*M* NaCl. *Xho*I-treated DNAs were end labeled by mixing 10 μl of the DNA digest with an equal volume of double-strength buffer (134 m*M* Tris, pH 8.8, 13.4 m*M* $MgCl_2$, 20 m*M* β-mercaptoethanol, 33.2

m*M* ammonium sulfate, 200 μg bovine serum albumin/ml, 0.33 m*M* each of dATP, dCTP, and dTTP) for bacteriophage T-4 DNA polymerase, 1 unit of T4 polymerase, and 10 μCi of deoxyguanosine [α-^{32}P]triphosphate. Incubation of the reaction mixture was carried out for 30 min to 3 hr. The samples were electrophoresed into a 0.8% agarose (SeaChem) slab gel. Gels were dried and autoradiography carried out.

RESULTS

Virus Isolated from IMC-Hz-1 Cell Culture Supernatant

The agent isolated from the IMC-Hz-1 cell culture supernatant is shown infecting a TN-368 cell in Fig. 1. The bacilli-form, enveloped nucleocapsids assemble in the nuclei. Preliminary ultrastructural studies did not produce evidence of virion-occluding crystalline structures typical of nuclear polyhedrosis or granulosis viruses. Infection of cultured cells culminate in lysis of both cell membrane and nuclear envelope with consequent release of enveloped virions into the culture medium either singly or in clumps.

The *Xho*I restriction enzyme fragment patterns of Hz-1V and HzSNPV DNAs are shown in Fig. 2. The dissimilarity of the two patterns and the scarcity of comigrating bands indicate strongly that the two viruses are separate entities. The Hz-1V DNA has been further characterized and will be the subject of a future report (Huang *et al.*, manuscript in preparation).

Plaque Assay for Hz-1V

Initial phase and electron-microscopic observation of lytic infection caused by Hz-1V in TN-368 cells suggested the fea-

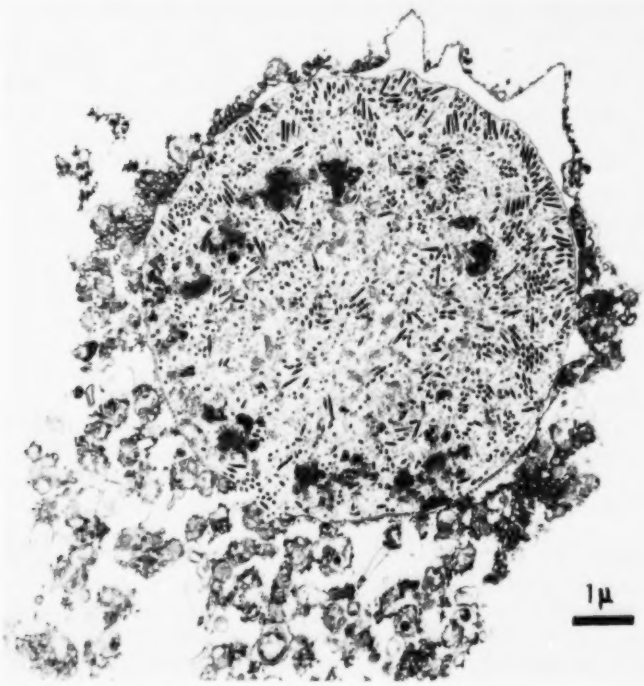

FIG. 1. Hz-1 virus-infected nucleus and appurtenant cytoplasm from a TN-368 cell. Note baculovirus morphology of Hz-1V and lytic nature of the infection.

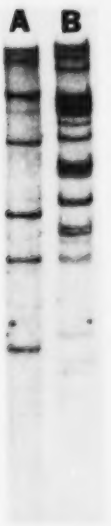

FIG. 2. Autoradiograms of *Xho*I restriction patterns of the DNAs of *Heliothis zea* S nuclear polyhedrosis virus (A) and Hz-1 virus (B) show the difference between the two agents. *Xho*I fragments were end labeled with deoxyguanosine [α-^{32}P]triphosphate and phage T4 DNA polymerase. The mixtures were electrophoresed into 0.8% vertical agarose slab gels, dried, and autoradiography carried out.

sibility of a plaque assay for infectivity titration. The plaques developed in a TN-368 monolayer are shown in Fig. 3. They were less opaque than the plaques produced by polyhedra-forming invertebrate viruses such as Ac*M*NPV E-2.

The dose response of the plaque assay is shown in Fig. 4. The mean number of plaques (squares), the calculated expected number of plaques (open circles), and the 95% confidence limits (bars) are indicated. The coefficient of determination, $r^2 = 0.99$, indicates minimal deviation from linear regression, and is evidence of good dose response.

Virus Growth Kinetics

The kinetics of a single cycle growth experiment in TN-368 cells with Hz-1V at an m.o.i. of 5 is shown in Fig. 5. During the initial 12 hr after inoculation, detectable infectious virus did not exceed the inoculum level. Subsequently, at 18 hr, the infectious titer showed a sharp rise to a level 10-fold higher than the inoculum. Released infectious virus peaked at 24 hr at 5×10^7 PFU/ml. This was followed by a steep fivefold drop in titer during the next 12-hr interval followed by a gradual decline within the next few days. Use of lower m.o.i.s (0.1 and 0.001) resulted (not shown) in higher titers and a concomitant m.o.i.-related shift of the peak to a later time.

Parallel cell viability studies (not shown) indicated that at the time of peak infectious titer, approximately 60% of the cell population was viable, as determined by trypan blue exclusion. However, even at 96 hr, mortality never reached 100%.

Susceptibility of Cell Lines to Hz-1V

Hz-1V infectivity to various cell lines was tested (Table 1). TN-368, Cl 10, Cl 13, and *M. brassicae* cells supported virus replication as evidenced by progeny virus titers well above that of the inoculum. A lower, but significant level of replication also occurred in two *S. frugiperda* cell lines, IPLB-21 and IPLB-21-1075. BTI-EAA and *M. sexta* cells neither developed CPE nor significant infectious titers, and appear not to support Hz-1V replication.

Comparative Efficiencies of TN-368 and Cl 13 As Indicator Cells in the Plaque Assay

Preliminary tests indicated that Cl 10 and Cl 13 cells exhibited more severe CPE than the parental TN-368 cell line after inoculation with Hz-1V from common preparations. These results suggested the possibility that the sensitivity of the plaque assay could be increased by utilization of these clonal cells for titration of virus. Clone 10, however, could not be used because the cells adhered poorly to the test dishes and confluent monolayers could not be maintained during assay manipulations. Cl 13 adhered more firmly, and with modifications, the plaque assay could be conducted without extensive disruption of the monolayer.

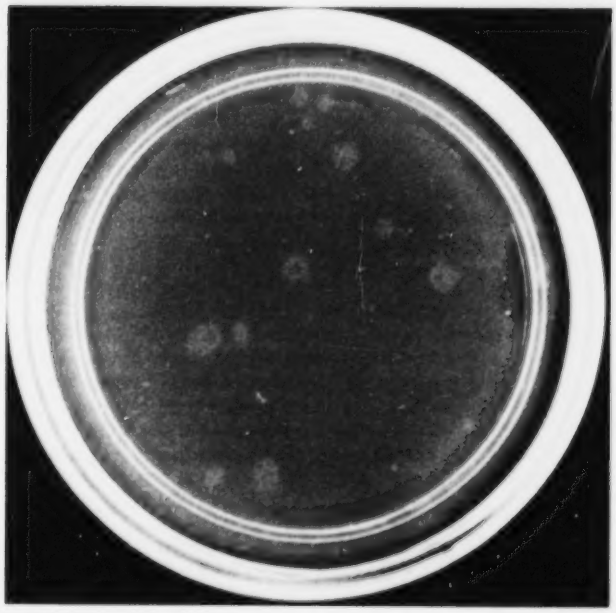

FIG. 3. Plaques formed by Hz-1 virus using TN-368 indicator cells. Four days postinfection at 28°.

The comparative efficiency of Cl 13 and TN-368 in assaying Hz-1V infectivity titers was tested by inoculating nearly confluent cultures of these two lines with aliquots from a common Hz-1V dilution series. Separate trials, using three different Hz-1V isolates showed Cl 13 cells consistently indicated a three- to fivefold higher titer than TN-368 cells (Table 2). The differences were statistically significant ($P < 0.05$). However, neither Cl 10 nor Cl 13 attached to assay dishes as well as the parental cell line, and could not be easily utilized in the plaque assay.

Persistent Infections

During the process of plaque purification of Hz-1V for DNA characterization, some TN-368 cultures inoculated with single plaque isolates showed substantial decreases in CPE. Less than 50% of the cells lysed, and the remaining cells appeared healthy. Titration of clarified growth medium of these cultures indicated titers higher than that expected from the CPE observed. The cell population quickly grew to confluency. These cultures were subcultured, and the 4-day clarified growth media from each passage titered for infectivity. The results from a representative culture for the initial 3 months are shown in Fig. 6. The production of infectious virus dropped 100-fold between the first and eighth subculturings, but eventually, the 4-day growth media titers stabilized near a basal level of 1×10^4 PFU/ml. The titer was maintained near this level out to the 100th passage. Cultures such as A-186, shown in Fig. 6, generally appear healthy, grow well, and are morphologically indistinguishable from the line (TN-368) from which it was derived. It constantly sheds infectious virus.

An example of the release of infectious virus from A-186 cells into the medium during a passage is shown in Fig. 7. Cells were seeded at a concentration of 2×10^5 cells/ml in fresh medium and after 1, 6, 18, 24, 36, 48, 72, and 96 hr, the clarified

growth medium was assayed for infectivity. A slight drop in titer 6 hr after subculturing was followed by a generally increasing titer which peaked at 2×10^4 PFU/ml at 96 hr. Distinct bursts of infectious Hz-1V similar to that observed with infected normal TN-368 cells were not observed.

Usually, before the 25th subculturing, persistently infected cultures go through one or more infectious crises as was evidenced by the sudden development of extensive CPE. During these periods, detectible infectious virus increased about 100-fold in those cases where the media were assayed. The cells which survive the crises continue to divide and eventually the culture recovers. A basal infectious titer of about 1×10^4 PFU/ml 4 days after subculturing is then reestablished.

Resistance of Persistently Infected Cultures to Homologous Superinfection

Certain types of persistent infections result in resistance to superinfection by homologous virus while retaining their susceptibility to infection by heterologous viruses (Joklik, 1977). To determine whether cultures constantly shedding Hz-1V possessed this property, the persistently infected HA-38 cell line and normal TN-368 cells were inoculated with homologous Hz-1V or heterologous Ac*M*NPV E-2, which replicates well in the TN-368 cell line. Periodically after infection or superinfection, clarified growth medium was titered by the plaque assay for infectious virus. Because the quantities of virus normally released by the persistently infected

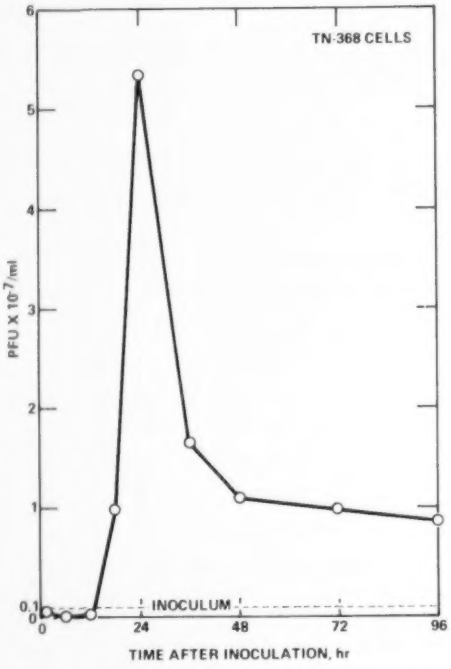

FIG. 5. Release of infectious Hz-1 virus into the culture medium after inoculation of log phase TN-368 cells at a multiplicity of 5. This single burst experiment shows the replication time for this virus is approximately 24 hr. Culture flasks (25 cm^2) were prepared and divided into two groups. One group was inoculated with Hz-1V and the remainder with 1 ml of medium. An inoculated and control flask were removed from the 28° incubator after the periods indicated in the figure. The culture media were centrifuged to remove cells and heavy debris then frozen at −85°. The infectious titers of these samples were later determined with the plaque assay.

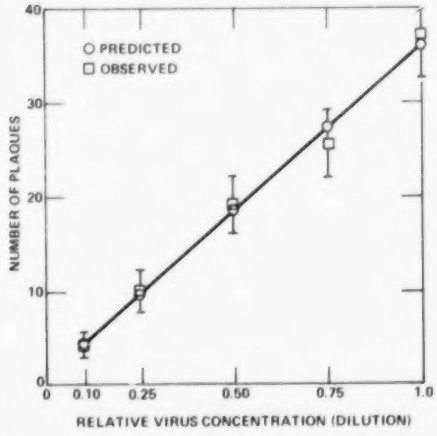

FIG. 4. Dose response of plaque assay for Hz-1 virus using TN-368 indicator cells. The assay shows a good dose response with minimal deviation from linear regression ($r = 0.99$). The squares represent the mean number of plaques 4 days after inoculation at 28° from eight replicate cultures and the open circles represent the calculated expected values. The bars indicate the 95% confidence limits.

TABLE 1

SUSCEPTIBILITY OF CELL LINES TO Hz-1 VIRUS INFECTION

Cell line	Infectious titer (PFU/ml × 10^{-4})			Effect
	Inoculum[a]	2-day medium	5-day medium	
TN-368	1.0	520.0	1000.0	CPE[b]
Cl 10	1.5	0.7	200.0	CPE
Cl 13	1.8	0.7	100.0	CPE
M. brassicae IPLB-21 (1075)	1.0	50.0	NT[c]	CPE
	2.5	1.0	10.0	Mild CPE and clumping
IPLB-21	2.6	1.0	40.0	CPE
BTI-EAA	1.0	2.3	NT	Healthy
M. sexta	1.0	2.0	NT	Healthy

[a] Approximate multiplicity of infection of 0.1.
[b] Cytopathic effects.
[c] Not tested.

HA-38 cells might mask low levels of superinfection, untreated cultures of both the persistently infected cell line and the TN-368 cell line were run as controls and assayed at the same time. Figure 8 shows the results of this experiment. Ac*M*NPV E-2 replicated similarly in both TN-368 and HA-38 cells, as evidenced by nearly identical titers of released infectious virus at various intervals after inoculation. In contrast, challenge by homologous Hz-1V resulted in substantial replication in TN-368 cells, but not in HA-38 cells, which did not produce titers above the inoculum level and appeared to be resistant to superinfection. Cytopathic changes mirrored the differences shown by the infectious titers. HA-38 cultures superinfected with

TABLE 2

COMPARATIVE EFFICIENCY OF TN-368 AND Cl 13 AS INDICATOR CELLS FOR THE TITRATION OF Hz-1 VIRUS[a]

Experiment	Number of replicates	Indicated titer (log PFU/ml)	
		TN-368	Cl 13
A	12	5.98 ± 0.15	6.56 ± 0.10*
B	13	5.82 ± 0.22	6.54 ± 0.06*
C	24	6.87 ± 0.19	7.10 ± 0.24*

[a] Plaque assays were conducted using replicate monolayers of each cell type concurrently inoculated with a common Hz-1 virus preparation. Experiments A, B, and C were conducted with different Hz-1 virus isolates.

* Significantly different at $P < 0.05$ using Dunnett's test.

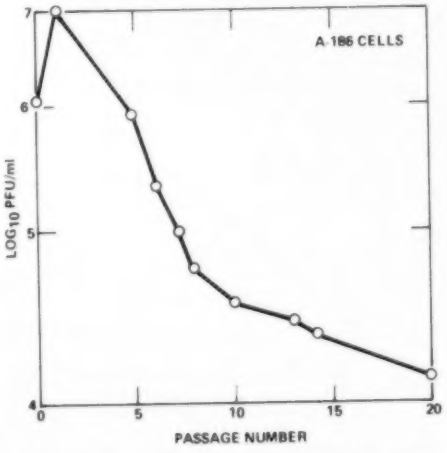

FIG. 6. Titers of Hz-1V produced by A-186 cultures during 20 continuous passages after establishment of persistent infection by high multiplicity inoculation of a TN-368 culture. The titers are of the media obtained 4 days after subculturing during preparation of cells for passage. Titers remained near 1 × 10^4 PFU/ml even at the 100th passage.

Hz-1V showed little or no detectable CPE, whereas the TN-368 culture developed the usual severe CPE. Endogenous virus levels in the untreated HA-38 persistently infected cultures were not high enough to interfere with the experiment. The maximum background titer was 3.7×10^4 PFU/ml, or 7.4% of the superinfected titer average. As expected, control TN-368 cells had no measureable infectious titer.

Induction of Persistently Infected Cell Cultures

The first persistently infected TN-368 cultures were encountered during propagation of Hz-1V isolates from single plaques. To determine whether persistently infected cultures could be reproducibly obtained, deliberate inductions were attempted in TN-368 cells. Cells were infected with Hz-1V at an m.o.i. of 5, and 4 days later when CPE reached its maximum of about 95%, fresh culture medium was replenished every 3 or 4 days, and the growth of the surviving cells became apparent after about 2-3 weeks. By this method, persistently infected TN-368 cultures were obtained on numerous occasions.

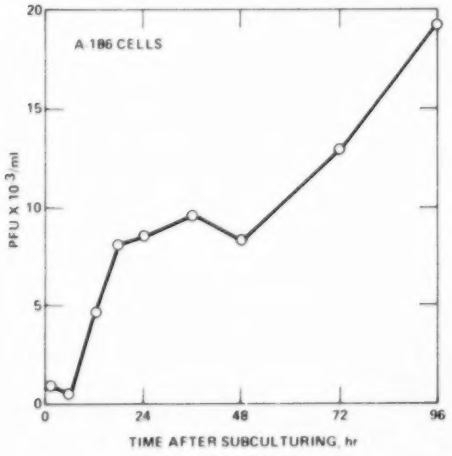

FIG. 7. Infectious virus released into the growth medium by persistently infected A-186 cells within a passage at intervals after subculturing. Replicate A-186 cultures in the 20th passage were prepared. After incubation at 28° for the periods indicated in the figure, sample cultures were removed, media clarified by centrifugation and frozen at −85°. The samples were later plaque assayed for Hz-1V infectivity.

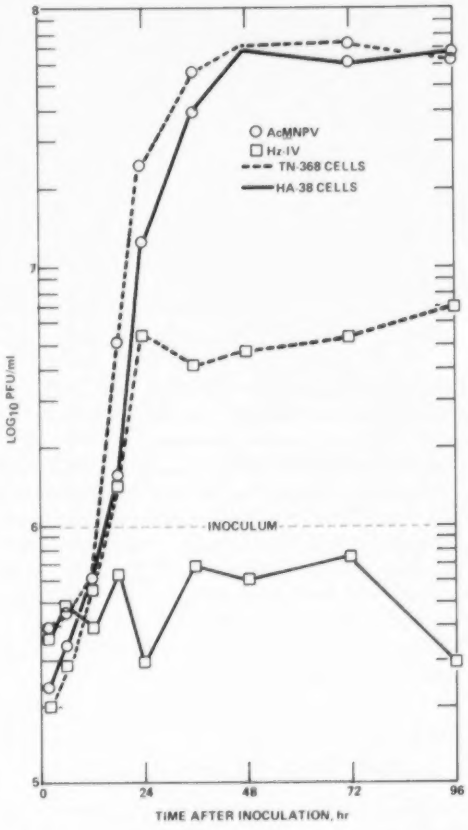

FIG. 8. Susceptibility of normal TN-368 (broken lines) and persistently infected HA-38 (solid lines) cultures to homologous Hz-1 (squares) and heterologous *Autographa californica M* nuclear polyhedrosis virus (Ac*M*NPV) isolate E-2 (circles). Heterologous virus replicates with identical efficiency in both types of cultures but persistently infected cells appear resistant to superinfection by the homologous Hz-1 virus. Cultures of TN-368 or Ha-38 cells in logarithmic growth were inoculated with Hz-1V, Ac*M*NPV-E2, or sterile culture media. After predetermined incubation periods at 28° sample cultures of each cell-inoculum combination were removed from the incubator, the media centrifuged to remove cells and large debris, and stored at −85°. Each sample was later titered for infectivity by plaque assay.

Earlier, it was shown that clones of TN-368 cells differed in their sensitivity to Hz-1V. To determine whether persistently infected culture formation was related to cell type, 10 separate cultures each of clone 10, clone 13, and parental TN-368 were inoculated with the same Hz-1V inoculum at an m.o.i. of 5 (relative to TN-368). Subsequently, at 4-day intervals, the cultures with living cells were pelleted and replenished with fresh medium. As shown in Table 3, all 10 of the TN-368 cultures produced persistently infected cells. All of the clone 13 cell cultures died after infection. Initially, 100% of the clone 10 cultures appeared to have died, but at 2 weeks postinoculation, some viable dividing cells were observed in 50% of the flasks. These cells grew very slowly, and a population level sufficient for subculturing was never obtained despite several months of nurturing. Thus, it appears, under the conditions of the experiment, only the uncloned parental TN-368 cell cultures were capable of establishing persistently infected cultures.

Electron Microscopy of Cells from Persistently Infected Cultures

Electron microscopic examination of cells from persistently infected cultures indicated only a small proportion of the cells exhibited signs of active viral replication. Preliminary ultrastructural comparison of infected cells from acutely and persistently infected cultures did not reveal prominent differences. However, both lysed cells and bare nuclei with and without signs of viral replication (Fig. 9) appeared to be more frequent in persistently infected cultures than in normal uninfected cultures.

TABLE 3

EFFECT OF CELL TYPE ON ESTABLISHMENT OF PERSISTENT INFECTION BY Hz-1 VIRUS

Cell line	No. cultures	Percentage persistently infected[a]
TN-368	10	100
Cl 10	10	0[b]
Cl 13	10	0

[a] Log phase cultures were infected with Hz-1 virus at a multiplicity of 5.

[b] 50% of the cultures contained surviving cells after the initial infection which were maintained for several months, but normally growing cultures were never established.

DISCUSSION

Hz-1V appears to be very similar in morphology and host range to the agent isolated by Granados *et al.* (1978). The bacilliform virion is composed of an enveloped rod-shaped nucleocapsid, which is typical of a baculovirus. Virion-occluding proteinaceous bodies characteristic of most baculoviruses were not observed during electron microscopic examination of Hz-1V-infected cells. The DNA is double stranded, and restriction enzyme fragment pattern shows Hz-1V and Hz-*S*NPV, the active ingredient of a registered biorational pesticide, to be different.

The lytic nature of Hz-1V infections in TN-368 cells permitted successful development of a plaque assay. Hz-1V produces more translucent plaques than the refractile inclusion body-filled ones of Ac*M*NPV E-2. Titrations with this assay exhibited a good linear dose response which indicates that single Hz-1V particles initiate infections (Dulbecco and Ginsberg, 1973). Though Cl 13 was three to five times more efficient as indicator cells than the parental TN-368 they were not used routinely because they attached poorly to assay dishes. Volkman and Summers (1975) found uncloned TN-368 to be more sensitive than Cl 13 in plaque assays for Ac*M*NPV.

Infectivity titrations of media after various intervals following high multiplicity infection of TN-368 cultures indicated that Hz-1V has a 24-hr replication cycle at 28°. Peak titer was followed by a sharp drop in infectivity. Some reasonable possibilities that may contribute to this phenomenon are degradation or aggregation of virions, adsorption of progeny particles by remaining cells, and the release of factors

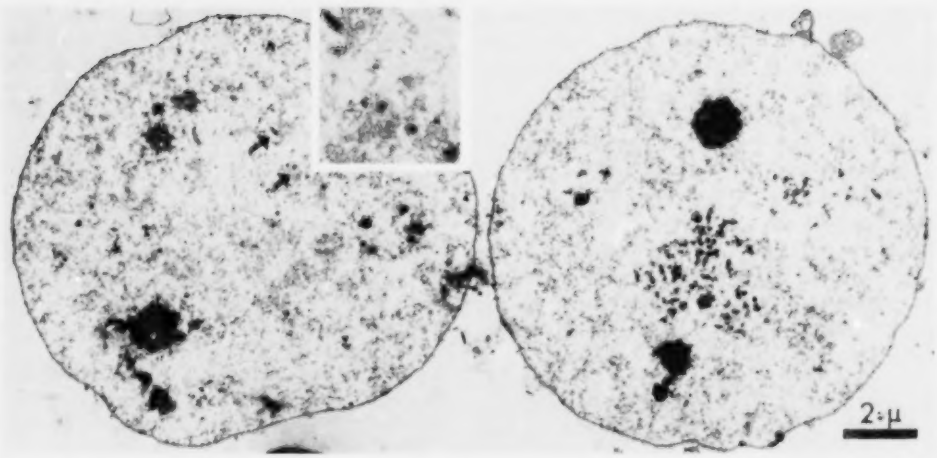

FIG. 9. Free nuclei and adherent cytoplasmic structures from Hz-1 virus persistently infected cultures. Such cultures contained a higher frequency of cells showing cell membrane and/or nuclear envelope lysis than normal TN-368 cultures. Note the presence of Hz-1 virions (arrows and inset) in one nucleus.

that interfere with plaque formation during infectivity titrations.

Although Hz-1V was isolated from a persistently infected cell line, its propensity to reestablish persistent infections in TN-368 was surprising. These cultures exhibit characteristics common to other persistent infections (Joklik, 1977) and, specifically, to those established and modulated by defective interfering particles (Huang and Baltimore, 1970; Nayak, 1980). Persistently infected cultures continually shed infectious Hz-1V even after extensive subculturing. During early passages many such cultures go through sudden acute infectious crises followed by recovery. Resistance to superinfection by Hz-1V was also demonstrated for persistently infected cultures. Though such specific interference can arise from other virus-related factors (Lee and Schloemer, 1981) recent observations on Hz-1V preparations containing interfering factor(s) demonstrated the predominance of physically altered particles and DNA over normal ones (Kawanishi *et al.*, manuscript in preparation). Present evidence also implicates the function of cellular determinants in the establishment of persistent infections by Hz-1V. Under the conditions employed, persistent infections could only be established in uncloned parental TN-368 cultures and not in clones derived from it (Cl 10, Cl 13).

Earlier it was noted that Cl 13 cells were also more sensitive plaque indicators than TN-368. Although this finding appears discordant with the lower productivity of Hz-1V infection in Cl 13 cultures compared to those of TN-368 in the host range studies (Table 1), this experiment was neither replicated extensively nor were experimental conditions controlled as rigorously as in the previous experiment (Table 2). Therefore, quantitative interpretations permitted by the host range experiments are limited and may not be representative of the general case. Nevertheless, Hz-1V infections of cells like those of Cl 13 may produce fewer defective particles and consequently, cultures may be less prone to becoming persistently infected. This is not inconsistent with the observed influence of host cell on the generation and amplification of defective interfering particles of certain viruses (Crumpton *et al.*, 1981).

Direct electron microscopic examination of cells from persistently infected cul-

tures shows only a very low proportion of cells exhibiting signs of virus replication. Qualitative differences in the appearance of infected cells from persistently and acutely infected cultures were not evident in preliminary studies. However, there appears to be a higher incidence of free nuclei and lysis of cell membrane and nuclear envelopes without evidence of viral presence. Cloning of persistently infected cells in the presence of antibody currently in progress indicates, however, that a high percentage of single cell isolates carry the viral genome.

Neither the original source nor natural host of Hz-1V is known, and Hz-1V has a wide host range among Lepidopterous cell lines as is evident from the present results and those of Granados *et al.* (1978). This agent initiates persistent infections in cell cultures with apparent facility, and such cultures are not easily recognized by routine examination. Hz-1V persistently infected cultures also permit replication of other baculovirus, such as Ac*M*NPV E-2, as efficiently as normal cells. Additionally, ongoing cell-cloning experiments with persistently infected cultures indicate relatively stable association between the Hz-1V genome and cells from persistently infected cultures. These characteristics of Hz-1V enhance its potential occurrence as an adventitious agent in preparations of invertebrate viruses used as pesticides. For these reasons, and because its health and ecological effects remain unassessed, Hz-1V is of concern to us. Further studies of Hz-1V and its persistent infections are in progress.

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